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Research Article

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Inhibitory Effect of a Biflavonoid Antioxidant (Kolaviron) on Benzoyl Peroxide Induced Free Radical Generation in Rat Skin

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Abstract This study evaluated the inhibitory effects of kolaviron, a biflavonoid from *Garcinia kola* seed, on benzoyl peroxide (BPO)-induced free radical generation in rat's skin. Adult male rats were administered with Group I: 0.2 ml acetone per animal as control, Group II: 100 mg of kolaviron/kg b.wt./0.2 ml acetone, Group III: 20 mg BPO/0.2 ml acetone per animal, Group IV: 100 mg of kolaviron/kg b.wt./0.2 ml acetone plus 20 mg BPO/0.2 ml acetone per animal. Skin of all the animals were removed quickly and processed for biochemical analysis. Benzoyl peroxide-treated rats showed ($P < 0.05$) decrease in vitamin C, E, glutathione (GSH) and total antioxidant capacity (TAC) but when it is co-administered with kolaviron the values of vit C, E, GSH and TAC significantly increases. The values of protein carbonyl (PC), lipid peroxide (LP), malondialdehyde (MDA), nitrite (NO_2), conjugated diene (CD) and hydrogen peroxide generation (H_2O_2) in rats treated with Benzoyl peroxide ($P < 0.05$) increases significantly but decreases as kolaviron was co-treated ($P < 0.05$). Taken together, kolaviron inhibited the adverse effects of BPO induce free radical generation.

Keywords Biflavonoid Antioxidant, Garcinia kola seed

Introduction

A free radical is any chemical species (molecule, molecular fragment, atom, compounds, ions etc) capable of independent existence possessing one or more unpaired electrons, an unpaired electron being one that is alone in an orbital [1]. Free radicals are involved in the normal biology of living organisms. They act as messengers for signal transduction and also affect gene expression [2]. Free radicals are also involved in the pathogenesis of several chronic diseases such as inflammation, neurodegenerative diseases, ageing, rheumatoid arthritis, heart diseases, cancer, hypertension, some metabolic diseases like atherosclerosis, diabetes, and so on [3]. There are several enzymes and biomolecules in the living organism which act as free radical scavengers. Further, several dietary supplements containing vitamins or polyphenols also play a significant role on free radicals scavenging [4].

Benzoyl peroxide (BPO) is a free radical generating compound as well as a strong oxidizing agent and is widely used in industries as a polymerization initiator, as a bleaching agent for flour cheese and as an additive in cosmetics and pharmaceuticals particularly it is used for the treatment of acne vulgaris [5-6]. In murine skin BPO has been shown to be an effective tumor promoter in animals treated with chemical carcinogens such as 7,12 dimethyl benz(a)anthracene, benzo(a)pyrene and *N*-methyl-*N*-nitro guanidine [7].

Garcinia kola belongs to the family of plants called Guttiferae and the genus Garcinia. The seed, commonly known, as 'bitter kola' is eaten by many and it is culturally acceptable in Nigeria. Extracts of the plant have been employed in the African herbal medicine for the treatment of ailments such as laryngitis, liver diseases, cough and hoarseness of voice [8]. Kolaviron (KV) is an extract from the seeds of Garcinia kola, containing a complex mixture of biflavonoids and polyphenols namely Garcinia biflavonoid 1 (GB1), Garcinia biflavonoid 2 (GB2) and Kolaflavanone [9]. Many studies have confirmed the antioxidative, anti-lipid peroxidation, chemoprevention in colon carcinogenesis and anti-inflammatory effects of kolaviron in chemically-induced toxicity, animal models of diseases and in cell culture [10-13].

Research shows that hepatoprotective effects have been associated with plant extracts that are rich in phenolic compounds. However, to the best of our knowledge, the hepatoprotective effects of kolaviron, against benzoyl peroxide-induced free radicals generation in murine skin has not been demonstrated. The present study focused on evaluating the potential effects of kolaviron on benzoyl peroxide-induced free radicals generation in murine skin.

Materials and Methods

Chemicals

Animals

Four weeks old male albino rats Wistar strain (100–125 g) were obtained from Central Animal House facility of Niger Delta University, Nigeria and were housed in a ventilated room at 30 ◦C under a 12 h light–dark cycle. The rats were allowed to acclimatize for 1 week before the study and had free access to standard laboratory feed and water supply. The dorsal skins of the rats were shaved with an electric clipper (Whal).

Treatment Schedule

20 Wistar rats were randomly taken in five per cage and topical application was done in the following order

Group I: 0.2 ml acetone per animal as control

Group II: 100 mg of kolaviron/kg b.wt./0.2 ml acetone

Group IlI: 20 mg BPO/0.2 ml acetone per animal

Group IV: 100 mg of kolaviron/kg b.wt./0.2 ml acetone plus 20 mg BPO/0.2 ml acetone per animal

All the animals were sacrificed after overnight fasting by cervical dislocation 5 days after treatment. Skin of all the animals were removed quickly and processed for biochemical analysis.

Determination of Protein

The protein concentration in all samples was determined by the method of Lowry et al. 1951 using BSA as standard [14].

Hydrogen Peroxide Generation

The method of Wolff (1994) was utilized, phosphate buffer 2.5 ml 0.1 M pH 7.4, Ammonium Ferrous Sulfate 250 µL 0.25 mM, sorbitol 100 µL 0.1 M, xylenol orange 100 µL 0.025 M, H_2SO_4 25 µL 25 mM and 100 µL of skin homogenate was vortexed and incubated at room temperature for a minimum of 30 minutes. After which the reaction mixture was shaken to ensure proper mixing and then incubated for 30 minutes before reading at 560 nm. The concentration of the hydrogen peroxide generated was extrapolated from a standard curve and expressed as nmol H_2O_2/mg protein [15].

Determination of Protein Carbonyl (PC)

Protein carbonyl contents were determined according to the methods of Uchida and Stadtman, 1993. The skin homogenate sample was treated with an equal volume of 0.1% (w/v) 2,4-dinitrophenyl hydrazine in 2N HCl and incubated for 1 h at room temperature and then treated with 20% TCA. After centrifugation, the precipitate was washed three times with ethanol-ethyl acetate and dissolved in 8M guanidine hydrochloride in 133 mM Tris solution containing 13 mM EDTA. The absorbance was recorded at 365 nm. The results were expressed as nmol/mg protein based on the molar extinction coefficient of 22,000 M^{-1} cm^{-1} for aliphatic hydrazones [16].

Total Antioxidant Capacity measurements (TAC)

A colorimetric method using a Randox Assay Kit (Randox Laboratories Ltd, Antrim, UK) was used to measure the TAC. The assay is based on the incubation of supernatant of skin samples with 2, 2'-azino-di-[3-ethylbenzthiazoline

sulphonate 6 diammonium salt (ABTS) with a peroxidase (methmyoglobin) and hydrogen peroxide to produce the radical cation $ABTS^+$ which has a relatively stable blue-green color that is measured at 600 nm. Antioxidants present in the assayed plasma samples may or may not inhibit the oxidation of ABTS to $ABTS^+$ (cause suppression of the color production) to a degree that is proportional to their concentration. The capacity of the assayed sample antioxidants was compared with that of standard Trolox, a water soluble tocopherol analogue, which is widely used as a traditional standard for TAC measurement assays, and the assay results were normalized and reported as Trolox equivalent antioxidant capacity (TEAC) and defined as the nanomolar concentration of the Trolox antioxidant capacity of a calibration curve.

Glutathione Content (GSH)

Reduced glutathione was determined by the method of Jollow et al. 1974. One milliliter sample of skin homogenate was precipitated with 1.0 ml of sulfosalicylic acid (4%). The samples were kept at 4 ◦C for 1 h and then centrifuged at 1200 × *g* for 20 min at 4 ◦C. The assay mixture contained 0.4 ml supernatant, 2.6 ml sodium phosphate buffer (0.1 M, pH 7.4) and 0.2 ml DTNB (100 mM) in a total volume of 3.0 ml. The yellow colour developed, was read immediately at 412 nm on a spectrophotometer. Values were expressed as nmol GSH/mg protein [17].

Vitamin C

Vitamin C was determined by the method of Omaye et al 1979. Briefly, 0.5 ml of skin homogenate was mixed thoroughly with 0.5 ml of water, 1.0ml of 10% TCA and centrifuged at 3500×*g* for 20min. 1.0ml of the supernatant was subsequently treated with 0.2 ml of 75 µl of DTC reagent (2 g of dinitrophenyl hydrazine, 230 mg thiourea and 270 mg $CuSO4.5H_2O$ in 100 ml 5M H_2SO_4) and incubated at 37 ◦C for 3 h. Then 1.5ml of 65% sulphuric acid was added mixed well and the solution was allowed to stand at room temperature for another 30 min. The colour developed was read at 520 nm. Vitamin C levels were expressed as mg/mg protein from a standard [18].

Vitamin E

Vitamin E was estimated by the method of Desai, 1971. 1.0 ml tissue homogenate and 1.0 ml ethanol were thoroughly mixed, followed by the addition of 3.0 ml of petroleum ether, shaken rapidly and centrifuged. 2.0 ml supernatant was evaporated to dryness and 0.2 ml of 0.2% bathophenanthoraline was added. The assay mixture was protected from light and 0.2 ml of ferric chloride (0.001 M) was added followed by 0.2 ml of O-phosphoric acid (0.001 M). The total volume was made up to 3.0 ml with ethanol and the color developed was read at 530 nm. The vitamin E content was expressed as mg/mg protein [19].

Lipid Peroxide Determination (LP)

FOX 2 method was used to measure the levels of lipid hydroperoxides (LOOH) in plasma samples [20-21]. FOX 2 Reagent was prepared by mixing following reagents: (i) 0.1 ml of 2.5 mM ammonium ferrous sulphate (dissolved in 35.71 mM H_2SO_4), (ii) 0.6 ml of 35.71 mM H_2SO_4, (iii) 0.1 ml of 1 mM xylenol Orange and (iv) 0.1 ml distilled water. To 0.9 ml of FOX 2 reagent 100 µl of plasma sample was added, vortexed and incubated at room temperature for 1 hour. Then it was centrifuged to remove any flocculated materials and absorbance of supernatant was read at 552 nm against a blank. Cumene hydroperoxide was taken as the standard and a linear curve was obtained in the concentration range of 25-200 nmols/ml. The LOOH content was expressed as nmol lipid hydroperoxide/mg protein.

Determination of Conjugated Diene

Tissue conjugated diene (CD) were analyzed by the method of Recknagel and Glende, 1984. Lipids were extracted from tissue samples by using chloroform/methanol (2:1) mixture, dried under nitrogen atmosphere, dissolved in cyclohexane and measured spectrophotometrically at 232 nm (Perklin Elmer UV/VIS Spectrometer). The results were expressed as mmol/mg protein [22].

Measurement of Malondialdehyde (MDA)

Lipid peroxidation as evidenced by the formation of thiobarbituric acid reactive substances (TBARS) was estimated by the method described by Ohkawa et al., 1979. To 0.2 ml of tissue homogenate, 0.2 ml of 8.1% Sodium dodecylsulphate, 1.5ml of 20% acetic acid and 1.5ml of 0.8% TBA were added. The mixture was made up to 4.0 ml with distilled water and then heated in a boiling water bath at 95°C for 60 min. After cooling, 1.0 ml water and 5.0 ml n-butanol/pyridine mixture were added and shaken vigorously. After centrifugation at 600×g for 10 min, the pink

coloured chromogen formed by the reaction of 2-thiobarbituric acid with the breakdown products of lipid peroxidation was read at 535 nm. The results were expressed as the nmol MDA formed/mg protein by using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} [23].

Nitrite Determination

Nitrite assay was done using Griess reagent by the method of Green et al. 1982. In brief, 100 µl of Griess reagent (1:1 solution of 1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride in water) was added to 100 µl of skin homogenate was incubated for 5–10 min at room temperature protected from light. Purple/magenta color began to form immediately. Absorbance was measured at 546 nm, nitrite concentration was calculated using a standard curve for sodium nitrite, and nitrite levels were expressed as nmol/mg protein [24].

Statistical Analysis

The level of significance between different groups is based on Dunnett's t-test, followed by the analysis of variance test. The P-values of less than 0.05 have been taken as significant.

Results

Table 1: Effect of treatment of animals with garcinia kola on BPO mediated free radicals biomarkers

Parameters	Group I	Group II	Group III	Group IV
PC (nmol/mg protein)	2.64±0.17[a]	2.48±0.12 [a]	9.66±0.46[b]	3.02 ± 0.26[c]
LP(nmol/mg protein)	3.49±0.54 [a]	3.82±0.55 [a]	10.68±1.71[b]	4.39 ± 1.45[c]
MDA (nmol/mg protein)	2.76±0.56 [a]	2.89±0.43 [a]	10.88±0.39[b]	4.26 ± 0.42[c]
Nitrite (nmol/mg protein)	5.67±2.31 [a]	6.35±1.89 [a]	17.56±3.12[b]	8.92 ± 1.28[c]
H_2O_2(nmol/mg protein)	21.18±1.42 [a]	22.38±1.29 [a]	38.90±0.94[b]	25.25 ± 1.46[c]
CD (nmol/mg protein)	1.41±0.09 [a]	1.79±0.43 [a]	6.39±1.15[b]	2.35 ± 0.54[c]

Protein carbonyl (PC), Lipid peroxide (LP), Malondialdehyde (MDA), Conjugated diene (CD)

Values are mean ± SD for groups of five rats each. Values are statistically significant at $P < 0.05$. Values in a row not sharing a common superscript letter (a–c) differ significantly.

Table 2: Effect of treatment of animals with garcinia kola on BPO mediated antioxidants biomarkers

Parameters	Group I	Group II	Group III	Group IV
Vit. E	1.26±0.92[a]	1.48±0.73[a]	0.24±0.05[b]	1.18±0.41[c]
Vit. C	1.50±0.12[a]	1.64±0.07[a]	0.18±0.06[b]	1.83±0.055[c]
GSH	8.22±0.52[a]	8.24±0.42[a]	2.18±0.59[b]	7.02±0.21[c]
TAC	87.84±2.50[a]	86.87±2.08[a]	48.70±2.95[b]	62.5±3.18[c]

Vitamin E, C (vit. E, C) mg/mg protein, Glutathione (GSH) nmol GSH/mg protein, Total Antioxidant Capacity (TAC) nmol TEAC

Values are mean ± SD for groups of five rats each. Values are statistically significant at $P < 0.05$. Values in a row not sharing a common superscript letter (a–c) differ significantly.

The results of table 2 show significant decrease in the level of antioxidants Vit C, Vit E, GSH and total antioxidant capacity (TAC)caused by benzoyl peroxide treatment.The reduced form of glutathione is GSH, glutathione, whilst the oxidized form is GSSG, glutathione disulphide. The antioxidant capacity of thiol compounds is due to the sulphur atom, which can easily accommodate the loss of a single electron [1]. Vitamins E are organic compounds found in the diet necessary for normal fish growth. Vitamins are divided into two groups; water-soluble and fat-soluble. Vitamin E is a fat-soluble vitamin existing in eight forms [25]. Each form has its own biological activity, with a specific potency or functional use in the body, Vitamin E is well known for its role as an antioxidant, and Alpha-tocopherol (α-tocopherol) is the most active form of vitamin E and a powerful biological antioxidant. Vitamin C is a monosaccharide antioxidant found in both animals and plants. As one of the enzymes needed to make ascorbic acid has been lost by mutation during human evolution, it must be obtained from the diet and is a vitamin. Most other animals are able to produce this compound in their bodies and do not require it in their diets [1].

Total antioxidant capacity in the skin homogenate measures the antioxidant potential in skin homogenate compared to a water soluble vitamin trolox termed trolox equivalent antioxidant capacity (TEAC). The vit C, E, GSH and TAC decreased significantly in the benzoyl peroxide treated group III as compared with the control group I this could be the same reason stated earlier that BPO can generate free radicals and a strong oxidizing agent [26]. Free radicals generated in group III rats reduces the antioxidants potential in the skin homogenate leading to low values of The vit C, E, GSH and TAC. Garcinia kola extractcontainsKolaviron (KV) a mixture of biflavonoids and polyphenols namely Garcinia biflavonoid 1 (GB1), Garcinia biflavonoid 2 (GB2) and Kolaflavanone [9]. These antioxidants are involve in scavenging free radicals and oxidants that could be part of the reasons why the values of vit C, E, GSH and TAC increases in group IV because kolaviron is also shown to modulate antioxidant enzymes [12]. These findings are in line with similar works of Rupjyoti et al., 2003 who reported the modulatory effect of Moringa Oleifera against7,12 dimethylbenz(a)anthracene (DMBA) induce Skin Papillomagenesis in Mice [27].

MDA formation is one of the important biomarkers of free radical generation, and high level of MDA formed has been observed after treatment with BPO [26]. The highly reactive hydroxyl radical attacks unsaturated lipid membranes and leads to the generation of the conjugated dienes, lipid peroxyl radical, which has an unpaired electron and further forms chain reaction in peroxidation of the lipid molecules. Consistent with earlier reports, our results also showed remarkable increase in the level of MDA, PC, H_2O_2, LP, NO_2 after BPO treatment. This is even in the group III as compared to normal control of group I, the values of these parameters increases due to BPO effect. Nitrite content is an indirect measurement of nitric oxide and protein carbonyl content, hydrogen peroxide generation, lipid peroxide and malondialdehyde all increases in group III but decreases in the garcinia kola extract treated group as compared with group III. These results are in consonant with the recent findings of [26] who reported the ameliorative effect of 13-*cis* Retinoic acid against BPO induce oxidative stress and hyperproliferative response in murine skin.

In conclusion, the findings of the present study showed that garcinia kola extract supplementation effectively inhibited the free radical generation possibly by attenuating oxidative damage and scavenging of free radicals.

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